Characterization of Thermophilic Bacterial UvrD in *Escherichia Coli* Expression System

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Introduction

Research Summary

Background

- Defective mismatch repair (MMR) pathways caused by mutations in MMR proteins are identified in about 7% of all gastrointestinal cancers.[1]
- When DNA polymerase makes mistakes during DNA replication, the cell corrects these mistakes by recruiting the MMR pathway.[2]
- Understanding how MMR proteins function may help develop safe treatments for patients with MMR defective tumors.
- Helicases are essential for understanding MMR as they break the hydrogen bonds between double-stranded DNA. They interact with damaged and nicked DNA in an ATP-dependent manner to unwind segments of DNA to prepare for repair.[3]
- This research focused on *Thermus aquaticus* (*Taq*) UvrD, a homologue of a human helicase involved in MMR pathways, to elucidate how *Taq* UvrD binds to DNA, the factors affecting its enzymatic activity, and the directionality of its unwinding.[4]
- This research was expanded by studying other thermophilic helicases such as *Thermoanaerobacter tengcongensis* (*Tte*) UvrD.

Hypothesis

We predict that FRET analysis will conclude that *Taq* UvrD binds to ssDNA and moves from the 3' to the 5' end. This will be proven by an experiment showing that the ssDNA with the 3' overhang will show increased absorbance at 260 nm over time and an increase in emission from the donor molecule because it is no longer being accepted by its corollary acceptor.

Significance

- Characterizing *Taq* and *Tte* UvrD better informs how elements of the MMR pathway correct genetic mistakes, and has potential to be clinically applied in both cancer and genetic research.

Methods

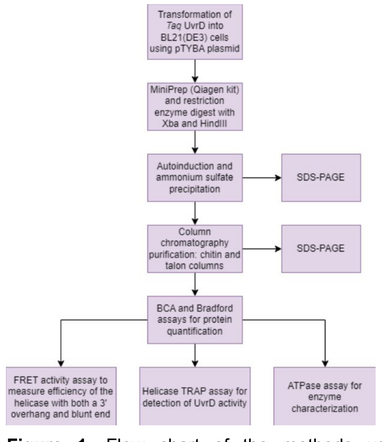

Figure 1. Flow chart of the methods used in the expression, purification, and characterization of thermophilic UvrD helicases.

- Restriction enzyme digest was used to determine the proper length for the vector and gene insert
- SDS-PAGE was used to determine purity of protein purification
- Helicase TRAP assay and FRET activity assay were used to determine helicase activity
- ATPase assay was done to determine enzyme kinetics with the Malachite Green Phosphate Assay Kit

Results

Figure 2. Investigating Properties of *Tte* UvrD

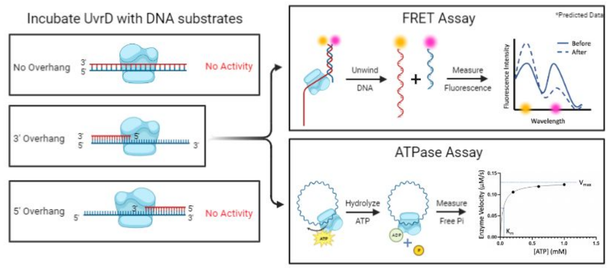

Figure 2. Testing the directionality and catalytic activity of UvrD using FRET and ATPase assays to determine key enzyme metrics

Figure 3. pTYBA plasmid expression of *Taq* UvrD and restriction enzyme digest

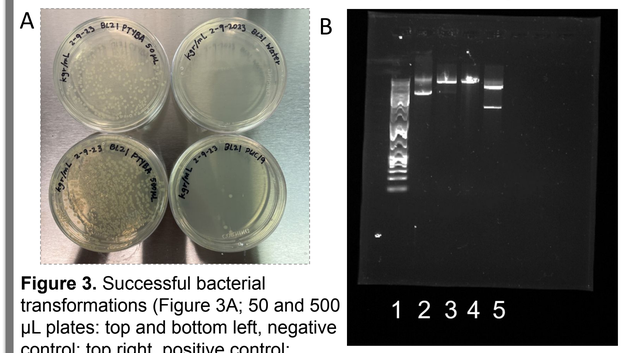

Figure 3. Successful bacterial transformations (Figure 3A; 50 and 500 µL plates: top and bottom left, negative control: top right, positive control: bottom right) and restriction enzyme digestion (Figure 3B) with XbaI (lane 3), HindIII (lane 4), and a double digest (lane 5). Uncut plasmid in lane 2 and ladder in lane 1.

Figure 4. Purification of *Taq* UvrD

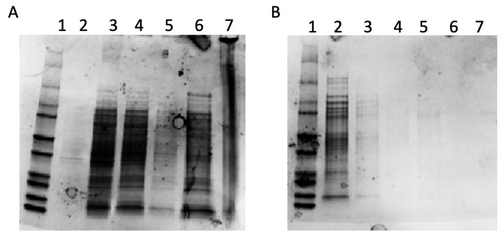

Figure 4. SDS-PAGE of ammonium sulfate precipitation (Figure 4A). Lane 1: protein molecular marker; lane 2: uninduced; lane 3: induced; lane 4: soluble; lane 5: insoluble; lanes 6-7: 40% ammonium sulfate supernatant and pellet. SDS-PAGE of column chromatography elution (Figure 4B). Lane 1: protein molecular marker; lanes 2-4: talon flow through, wash, elute; lanes 5-6: chitin flow through, wash; lane 7: pure protein. No purified protein was observed.

Figure 5. Helicase TRAP and FRET Activity Assays

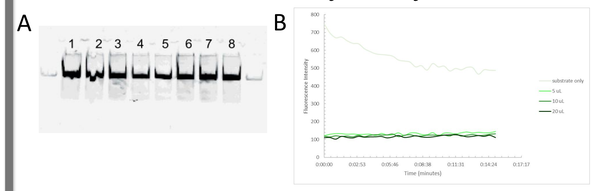

Figure 5. Helicase TRAP assay (Figure 5A) across four different incubation temperatures (room temperature, 46℃, 72℃, 90℃). Lanes 1, 3, 5, and 7 are controls at these temperatures, and 2, 4, 6, and 8 are experimental. FRET Activity (Figure 5B) measuring fluorescence of Cy5 after excitation of Cy3.

Amount *Tte* UvrD	FRET Efficiency (3' overhang)	Distance (nm) (3' overhang)	Δdistance (nm) (3' overhang)	FRET Efficiency (blunt end)	Distance (nm) (blunt end)	Δdistance (nm) (blunt end)
5 µL	0.692	4.54	-	0.521	5.13	-
10 µL	0.662	4.64	0.10	0.508	5.17	0.04
20 µL	0.597	4.87	0.23	0.501	5.20	0.03

Table 1. Calculation of FRET efficiency, distance, and change in distance for various concentrations of helicase. Substrate with both a 3' overhang and blunt end were tested. As concentration increased, efficiency decreased, indicating an increase in helicase activity.

Figure 6. *Tte* UvrD ATPase Activity

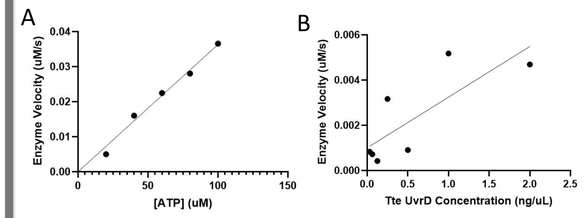

Figure 6 Measuring enzyme velocity as a function of ATP concentration (Figure 6A). V_{max} was determined to be 70.59 µM/s and K_m was determined to be 194.2 mM. Measuring the catalytic efficiency of *Tte* UvrD by varying *Tte* UvrD concentration (Figure 6B). k_{cat} was determined to be 0.0007807 s^{-1}.

- Although attempts to transform and digest *Taq* UvrD succeeded, purification post-ammonium sulfate precipitation by chitin and talon column chromatography did not.
- Characterization experiments using *Tte* UvrD concluded that there was helicase activity and that the FRET efficiency was greater with a 3' overhang compared to a blunt end. Absolute change in distance was also greater with a 3' overhang.
- Increased amount of helicase decreased efficiency and increased the change in distance between fluorophores, indicating more helicase activity.
- The maximum velocity of the enzyme was determined to be 70.59 µM/s with a K_m of 194.2 mM. The k_{cat} was 0.0007807 s^{-1} which is quite a low turnover number that results in a low catalytic efficiency (0.00402 $s^{-1}M^{-1}$).

Conclusions

- Despite difficulties in enzyme purification, this investigation of helicase protein behavior in thermophilic organisms yielded useful information on enzyme concentration, the efficiency with which *Tte* UvrD is able to unwind strands of DNA, the distance between fluorophores over time, the turnover number, and the initial enzyme velocity as a function of ATP concentration.
- All of these values can be used to make conclusions about *Tte* UvrD helicase behavior and possible contributions to the MMR pathway.
- This has many relevant clinical applications, particularly in MMR mutation-induced cancer research

Future Directions

- Repeat the attempts to purify *Taq* UvrD and thereafter the characterization experiments such as the FRET, helicase TRAP, and ATPase assays.
- The FRET assay controls showed significant photobleaching. In the future, we should reduce the time exposure to light sources to decrease the frequency of excitation-emission cycles.
- Repeat the ATPase experiment varying ATP with higher concentrations of ATP to ensure saturating conditions, as the linear line indicates this was not yet reached.
- Measure FRET activity with a 5' overhang to confirm the directionality of *Taq* and *Tte* UvrD.
- Complete hyperchromicity experiment.
- Compare *Tte* and *Taq* UvrD behavioral results to known helicases to make final conclusions.

Acknowledgements

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Citations

1. Preston, B. D.; Albertson, T. M.; Herr, A. J. DNA Replication Fidelity and Cancer. *Semin. Cancer Biol.* **2010**, *20* (5), 281–293.
2. Li, G.-M. Mechanisms and Functions of DNA Mismatch Repair. *Cell Res.* **2008**, *18* (1), 85–98.
3. Shukla, K., Thakur, R. S., Ganguli, D., Rao, D. N. & Nagaraju, G. Escherichia coli and Neisseria gonorrhoeae UvrD helicase unwinds G4 DNA structures. *Biochem. J.* **474**, (2017).
4. Pećina-Šlaus, N.; Kafka, A.; Salamon, I.; Bukovac, A. Mismatch Repair Pathway, Genome Stability and Cancer. *Front. Mol. Biosci.* **2020**, *7*, 122.